Attorney Docket No. 5051-574CT PATENT

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

In re: Martin et al.
Serial No.: 10/802,644
Filed: March 17, 2004
For: *Blocking Peptide for Inflammatory Cell Secretion*

Confirmation No.: 3963
Art Unit: 1644
Examiner: Haddad

Date: July 11, 2006

Mail Stop Amendment
Commissioner for Patents
P.O. Box 1450
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Attachment B

Exhibit 3

Li et al (2001), "MARCKS Protein is a Key Molecule Regulating Mucin Secretion by Human Airway Epithelia Cells *in Vitro*." J. Biol. Chem., Vol 276(44);40982-40990.

THE JOURNAL OF BIOLOGICAL CHEMISTRY Vol. 276, No. 44, Issue of November 2, pp. 40982–40990, 2001
Printed in U.S.A.

MARCKS Protein Is a Key Molecule Regulating Mucin Secretion by Human Airway Epithelial Cells *in Vitro**

Received for publication, June 18, 2001, and in revised form, August 30, 2001
Published, JBC Papers in Press, August 31, 2001, DOI 10.1074/jbc.M105614200

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Hypersecretion of airway mucin characterizes numerous respiratory diseases. Although diverse pathological stimuli can provoke exocytotic release of mucin from secretory cells of the airway epithelium, mechanisms involved remain obscure. This report describes a new paradigm for the intracellular signaling mechanism regulating airway mucin secretion. Direct evidence is provided that the myristoylated alanine-rich C kinase substrate (MARCKS) is a central regulatory molecule linking secretagogue stimulation at the cell surface to mucin granule release by differentiated normal human bronchial epithelial cells *in vitro*. Down-regulation of MARCKS expression or disruption of MARCKS function in these cells inhibits the secretory response to subsequent stimulation. The intracellular mechanism controlling this secretory process involves cooperative action of two separate protein kinases, protein kinase C and cGMP-dependent protein kinase. Upon stimulation, activated protein kinase C phosphorylates MARCKS, causing translocation of MARCKS from the plasma membrane to the cytoplasm, where it is then dephosphorylated by a protein phosphatase 2A that is activated by cGMP-dependent protein kinase, and associates with both actin and myosin. Dephosphorylated cytoplasmic MARCKS would also be free to interact with mucin granule membranes and thus could link granules to the contractile cytoskeleton, mediating their movement to the cell periphery and subsequent exocytosis. These findings suggest several novel intracellular targets for pharmacological intervention in disorders involving aberrant secretion of respiratory mucin and may relate to other lesions involving exocytosis of membrane-bound granules in various cells and tissues.

Mammalian airways are lined by a thin layer of mucus produced and secreted by airway epithelial (goblet) cells and submucosal glands. In diseases such as asthma, chronic bronchitis, and cystic fibrosis, hypersecretion of mucus is a common lesion. Excess mucus can contribute to obstruction, susceptibility to infection, and even to destruction of airway walls and contiguous tissues. The major components of mucus are mucin glycoproteins synthesized by secretory cells and stored within cytoplasmic membrane-bound granules. Upon appropriate stimulation, these granules are released via an exocytotic process in which the granules translocate to the cell periphery where the granule membranes fuse with the plasma membrane, allowing for luminal secretion of the contents.

Despite the obvious pathophysiological importance of this process, intracellular signaling mechanisms linking stimulation at the cell surface to mucin granule release have not been elucidated. It is known that a wide variety of agents and inflammatory/humoral mediators can provoke mucin secretion. These include cholinergic agonists, lipid mediators, oxidants, cytokines, neuropeptides, ATP and UTP, bacterial products, neutrophil elastase, and inhaled pollutants (reviewed in Refs. 1 and 2). Interestingly, many of these mucin secretagogues are also known to activate several protein kinases, and studies examining the regulation of excess secretion of mucin by airway epithelial cells from various species have consistently implicated involvement of either protein kinase C (PKC)[1] (3–6) or cGMP-dependent protein kinase (PKG) (7) in the secretory process. However, coordinated interactions or "cross-talk" between these two protein kinases in regulation of mucin secretion have not been demonstrated, nor have signaling events downstream of protein kinase activation that ultimately lead to the exocytotic release of mucin granules been elucidated.

Previously, we reported (8) development of a procedure to culture normal human bronchial epithelial (NHBE) cells in an air/liquid interface system in which the cells differentiate to a heterogeneous population containing secretory (goblet), ciliated, and basal cells that mimic their *in vivo* appearance and function. These cell cultures provide an ideal *in vitro* model system to study mechanisms regulating mucin secretion from human airway epithelium.

This report presents direct evidence demonstrating that the myristoylated alanine-rich C kinase substrate (MARCKS), a widely distributed PKC substrate for which a specific biological function has yet to be identified, is a key regulatory molecule mediating mucin granule release by NHBE cells. Secretion of mucin from these cells is maximized by activation of both PKC and PKG, and MARCKS serves as the point of convergence for coordinating the actions of these two protein kinases to control mucin granule release. The mechanism appears to involve PKC-

* This work was supported by Grant RO1 HL39862 from the National Institutes of Health and a grant from the state of North Carolina. The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked "*advertisement*" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

§ Performed portions of this work in partial fulfillment of the requirements for the degree of Doctor of Philosophy in Comparative Biomedical Sciences.

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[1] PKC, protein kinase C; PKG, cGMP-dependent protein kinase; PKA, protein kinase A; MANS, myristoylated N-terminal sequence; PMA, phorbol 12-myristate 13-acetate; PSD, phosphorylation site domain; 8-Br-GMP, 8-bromo-cyclic GMP; RNS, random N-terminal sequence; MARCKS, myristoylated alanine-rich C kinase substrate; NHBE, normal human bronchial epithelial; PP2A, protein phosphatase 2A; ELISA, enzyme-linked immunosorbent assay.

dependent phosphorylation of MARCKS, which releases MARCKS from the plasma membrane into the cytoplasm, where it is in turn dephosphorylated by a protein phosphatase 2A (PP2A) that is activated by PKG. This dephosphorylation would allow MARCKS to regain its membrane-binding capability (9–11), enabling its attachment to membranes of cytoplasmic mucin granules. In addition, MARCKS interacts with actin and myosin in the cytoplasm and thus could tether the granules to the cellular contractile apparatus, mediating subsequent granule movement and exocytosis.

EXPERIMENTAL PROCEDURES

NHBE Cell Culture—Expansion, cryopreservation, and culture of NHBE cells in the air/liquid interface were performed as described previously (8). Briefly, NHBE cells (Clonetics, San Diego, CA) were seeded in vented T75 tissue culture flasks (500 cells/cm^2) and cultured until cells reached 75–80% confluence. Cells were then dissociated by trypsin/EDTA and frozen as passage-2. Air/liquid interface culture was initiated by seeding passage-2 cells (2×10^4 cells/cm^2) in Transwell® clear culture inserts (Costar, Cambridge, MA) that were thinly coated with rat tail collagen, type I (Collaborative Biomedical, Bedford, MA). Cells were cultured submerged in medium in a humidified 95% air, 5% CO_2 environment for 5–7 days until nearly confluent. At that time, the air/liquid interface was created by removing the apical medium and feeding cells basalaterally. Medium was renewed daily thereafter. Cells were cultured for an additional 14 days to allow full differentiation.

Measurement of Mucin Secretion by ELISA—Before collection of "base line" and "test" mucin samples, the accumulated mucus at the apical surface of the cells was removed by washing with phosphate-buffered saline, pH 7.2. To collect the base-line secretion, cells were incubated with medium alone, and secreted mucin in the apical medium was collected and reserved. Cells were rested for 24 h and then exposed to medium containing the selected stimulatory and/or inhibitory reagents (or appropriate controls), after which secreted mucin was collected and reserved as the test sample. Incubation times for the base line and the test were the same but varied depending on the test reagent utilized. Both base line and test secretions were analyzed by ELISA using an antibody capture method described previously (12). The primary antibody for this assay was 17Q2 (Babco, Richmond, CA), a monoclonal antibody that reacts specifically with a carbohydrate epitope on human airway mucins (13). The ratio of test/base-line mucin, similar to a "secretory index" reported previously (14), was used to quantify mucin secretion, allowing each culture dish to serve as its own control and thus minimizing deviation caused by variability among culture wells. Levels of mucin secretion were reported as percentage of the medium control.

Radiolabeled Immunoprecipitation Assay—When labeling with [^{32}P]phosphate, cells were preincubated for 2 h in phosphate-free Dulbecco's modified Eagle's medium containing 0.2% bovine serum albumin and then labeled with 0.1 mCi/ml [^{32}P]orthophosphate (9000 Ci/mmol, PerkinElmer Life Sciences) for 2 h. For labeling with [^{3}H]myristic acid or ^{3}H-amino acids, cells were incubated overnight in medium containing 50 μCi/ml [^{3}H]myristic acid (49 Ci/mmol, PerkinElmer Life Sciences) or 0.2 mCi/ml [^{3}H]leucine (159 Ci/mmol, PerkinElmer Life Sciences) plus 0.4 mCi/ml [^{3}H]proline (100 Ci/mmol, PerkinElmer Life Sciences). Following labeling, cells were exposed to stimulatory reagents for 5 min. When an inhibitor was used, cells were preincubated with the inhibitor for 15 min prior to stimulation. At the end of the treatments, cells were lysed in a buffer containing 50 mM Tris-HCl (pH 7.5), 150 mM NaCl, 1 mM EDTA, 10% glycerol, 1% Nonidet P-40, 1 mM phenylmethylsulfonyl fluoride, 1 mM benzamidine, 10 μg/ml pepstatin A, and 10 μg/ml leupeptin. The radiolabeling efficiency in each culture was determined by trichloroacetic acid precipitation and scintillation counting. Immunoprecipitation of MARCKS protein was carried out according to the method of Spizz and Blackshear (15) using cell lysates containing equal counts/min. Precipitated proteins were resolved by 8% SDS-polyacrylamide gel electrophoresis and visualized by autoradiography. Anti-human MARCKS antibody (2F12) and nonimmune control antibody (6F6) used in this assay were provided by Dr. Perry Blackshear (NIEHS, Research Triangle Park, NC).

To assess MARCKS or MARCKS-associated protein complexes in different subcellular fractions, radiolabeled and treated cells were scraped into a homogenization buffer (50 mM Tris-HCl (pH 7.5), 10 mM NaCl, 1 mM EDTA, 1 mM phenylmethylsulfonyl fluoride, 1 mM benzamidine, 10 μg/ml pepstatin A, 10 μg/ml leupeptin) and then disrupted by nitrogen cavitation (800 pounds/square inch for 20 min at 4 °C). Cell lysates were centrifuged at 600 × *g* for 10 min at 4 °C to remove nuclei and unbroken cells. Post-nuclear supernatants were separated into membrane and cytosol fractions by ultracentrifugation at 400,000 × *g* for 30 min at 4 °C. The membrane pellet was solubilized in the lysis buffer by sonication. Immunoprecipitation was then carried out as described above.

MARCKS-related Peptides—Both the myristoylated N-terminal sequence (MANS) and the random N-terminal sequence (RNS) peptides were synthesized at Genemed Synthesis, Inc. (San Francisco, CA), then purified by high pressure liquid chromatography (>95% pure), and confirmed by mass spectroscopy with each showing one single peak with an appropriate molecular mass. The MANS peptide consisted of sequence identical to the first 24 amino acids of MARCKS, *i.e.* the myristoylated N-terminal region that mediates MARCKS insertion into membranes (9–11), MA-GAQFSKTAAKGEAAAERPGEAAVA (where MA = N-terminal myristate chain). The corresponding control peptide (RNS) contained the same amino acid composition as the MANS but arranged in random order, MA-GTAPAAEGAGAEVKRASAEAKQAF. The presence of the hydrophobic myristate moiety in these synthetic peptides enhances their permeability to the plasma membranes, enabling the peptides to be taken up readily by cells. To determine the effects of these peptides on mucin secretion, cells were preincubated with the peptides for 15 min prior to addition of secretagogues, and mucin secretion was then measured by ELISA.

Antisense Oligonucleotides—MARCKS antisense oligonucleotide and its corresponding control oligonucleotide were synthesized at Biognostik GmbH (Gottingen, Germany). NHBE cells were treated with 5 μM antisense or control oligonucleotide apically for 3 days (in the presence of 2 μg/ml lipofectin for the first 24 h). Cells were then incubated with secretagogues, and mucin secretion was measured by ELISA. Total RNA and protein were isolated from treated cells. MARCKS mRNA was assessed by Northern hybridization according to conventional procedures using human MARCKS cDNA (provided by Dr. Perry Blackshear, NIEHS, Research Triangle Park, NC) as a probe. MARCKS protein level was determined by Western blot using purified anti-MARCKS IgG1 (clone 2F12) as the primary detection antibody.

Transient Transfection—The phosphorylation site domain (PSD) of MARCKS contains the PKC-dependent phosphorylation sites and the actin filament-binding site (16). To construct a PSD-deleted MARCKS cDNA, two fragments flanking the PSD sequence (coding for 25 amino acids) were generated by polymerase chain reaction and then ligated through the *Xho*I site that was attached to the 5′-ends of oligonucleotide primers designed for the polymerase chain reaction. The resultant mutant cDNA and the wild-type MARCKS cDNA were each inserted into a mammalian expression vector pcDNA4/TO (Invitrogen, Carlsbad, CA). Isolated recombinant constructs were confirmed by restriction digests and DNA sequencing.

HBE1 is a papilloma virus-transformed human bronchial epithelial cell line (17) capable of mucin secretion when cultured in air/liquid interface. Transfection of HBE1 cells was carried out using the Effectene transfection reagent (Qiagen, Valencia, CA) according to the manufacturer's instructions. Briefly, differentiated HBE1 cells grown in air/liquid interface were dissociated by trypsin/EDTA and re-seeded in 12-well culture plates at 1×10^5 cells/cm^2. After overnight incubation, cells were transfected with the wild-type MARCKS cDNA, the PSD-truncated MARCKS cDNA, or vector DNA. Cells were cultured for 48 h to allow gene expression and then exposed to secretagogues and mucin secretion measured by ELISA. All transfections were carried out in the presence of pcDNA4/TO/*lacZ* plasmid (Invitrogen) (DNA ratio 6:1, total 1 μg DNA, ratio of DNA to Effectene reagent = 1:25) to monitor variations in transfection efficiency. Results showed no significant difference in β-galactosidase activities in cell lysates isolated from the transfected cells, indicating similar transfection efficiency among different DNA constructs (data not shown).

Protein Phosphatase Activity Assay—PP1 and PP2A activities were measured using a protein phosphatase assay system (Life Technologies, Inc.) as described (18) with modification. Briefly, NHBE cells were treated with 8-Br-cGMP or medium alone for 5 min. Cells were then scraped into a lysis buffer (50 mM Tris-HCl (pH 7.4), 0.1% β-mecaptoethanol, 0.1 mM EDTA, 1 mM benzamidine, 10 μg/ml pepstatin A, 10 μg/ml leupeptin) and disrupted by sonication for 20 s at 4 °C. Cell lysates were centrifuged and the supernatants saved for phosphatase activity assay. The assay was performed using ^{32}P-labeled phosphorylase A as a substrate. Released $^{32}P_i$ was counted by scintillation. The protein concentration of each sample was determined by the Bradford assay. PP2A activity was expressed as the sample total phosphatase activity minus the activity remaining in the presence of 1 nM okadaic acid. PP1 activity was expressed as the difference between the activities remaining in the presence of 1 nM and 1 μM okadaic acid, respectively.

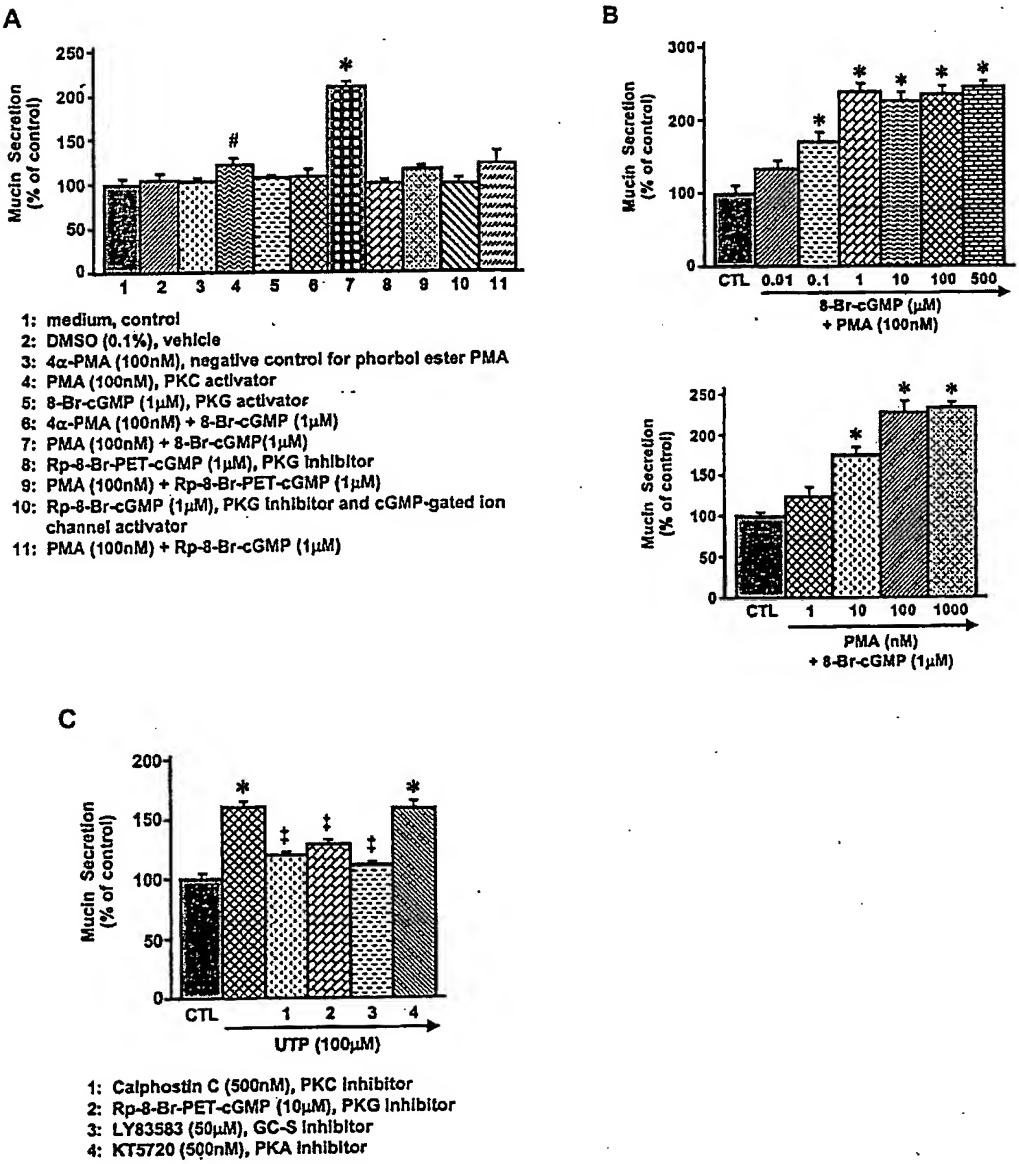

FIG. 1. **Mucin hypersecretion by NHBE cells is maximized by activation of both PKC and PKG.** *A* and *B*, NHBE cells were exposed to indicated reagent(s) or medium alone (*CTL*) for 15 min. *C*, NHBE cells were preincubated with the indicated inhibitor for 15 min and then stimulated with 100 μM UTP for 2 h. Secreted mucin in response to the treatment was collected and assayed by ELISA. Data are presented as mean ± S.E. ($n = 6$ at each point). *, significantly different from medium control ($p < 0.05$). #, different from medium control ($0.05 < p < 0.1$). ‡, significantly different from UTP stimulation ($p < 0.05$).

Protein phosphatase activities were reported as nmol of P_i released per min/mg total protein.

Cytotoxicity Assay—All reagents used in treating NHBE cells were examined for cytotoxicity by measuring the total release of lactate dehydrogenase from the cells. The assay was carried out using the Promega Cytotox 96 Kit according to the manufacturer's instructions. All experiments were performed with reagents at non-cytotoxic concentrations.

Statistical Analysis—Data were analyzed for significance using one-way analysis of variance with Bonferroni post-test corrections. Differences between treatments were considered significant at $p < 0.05$.

RESULTS

Mucin Hypersecretion from NHBE Cells Involves Activation of Both PKC and PKG

To determine the potential role of PKC and/or PKG in the mucin secretory process, NHBE cells were exposed to the following two specific protein kinase activators: the phorbol ester, phorbol 12-myristate 13-acetate (PMA), for activation of PKC, and the nonhydrolyzable cGMP analogue, 8-Br-cGMP, for activation of PKG. Preliminary studies examining mucin secretion in response to PMA stimulation at various concentrations for different times (up to 1 μM for 2 h) indicated that activation of PKC alone did not induce significant mucin secretion from NHBE cells, although a moderate secretory response was repeatedly observed at PMA concentrations higher than 100 nM ($0.05 < p < 0.1$). Also, the cells did not respond to the cGMP analogues at concentrations as high as 500 μM for up to 2 h of exposure. However, a combination of PMA + 8-Br-cGMP, affecting dual activation of PKC and PKG, provoked a rapid increase in secretion, approximately doubling it within 15 min of exposure (Fig. 1*A*). This secretory response induced by PMA + 8-Br-cGMP was concentration-dependent, with maximal stimulation at 100 nM PMA + 1 μM 8-Br-cGMP (Fig. 1*B*).

UTP is a well defined pathophysiologically relevant mucin secretagogue (19). Our preliminary studies showed that UTP (100 μM) could induce a significant increase in mucin secretion from NHBE cells after a 2-h exposure. To determine whether PKC and PKG were involved in regulation of mucin secretion in response to a pathophysiological stimulus, effects of PKC/PKG

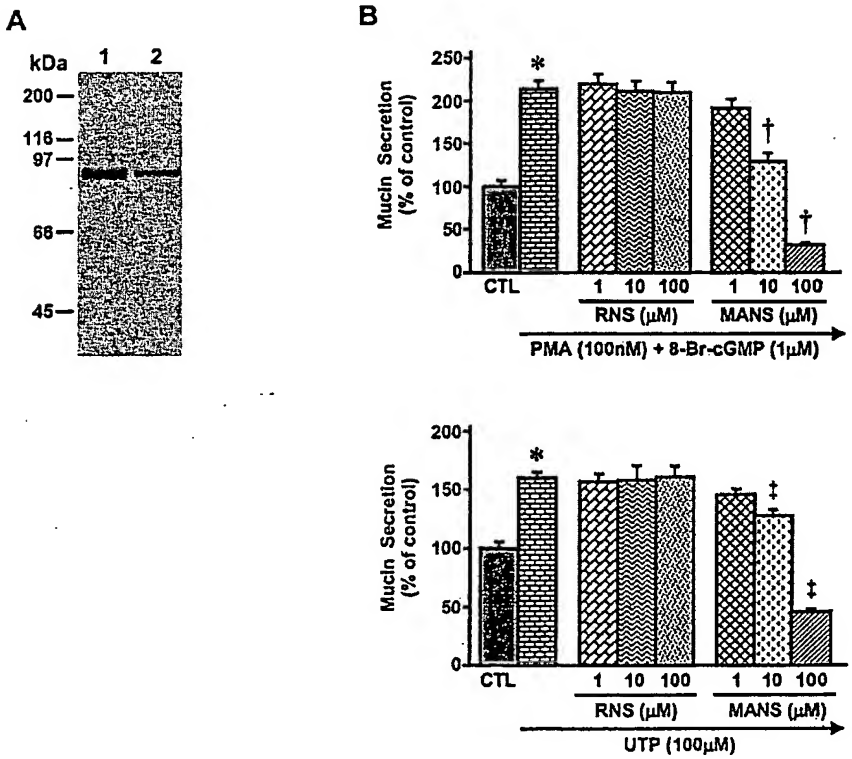

FIG. 2. **MARCKS protein is a key component of the mucin secretory pathway.** *A*, MARCKS is myristoylated and mostly membrane-associated in NHBE cells. Cells were labeled with [^{3}H]myristic acid overnight, and the membrane (*lane 1*) and the cytosol (*lane 2*) fractions were then isolated by differential centrifugation. MARCKS protein in each fraction was examined by immunoprecipitation as described. *B*, the MANS peptide blocks mucin hypersecretion induced by PMA + 8-Br-cGMP or UTP in a concentration-dependent manner. NHBE cells were preincubated with the indicated peptide for 15 min and then exposed to PMA (100 nM) + 8-Br-cGMP (1 μM) for 15 min or UTP (100 μM) for 2 h. Mucin secretion was measured by ELISA. Data are presented as mean ± S.E. (n = 6 at each point). *, significantly different from medium control ($p < 0.05$); †, significantly different from PMA + 8-Br-cGMP stimulation ($p < 0.05$); ‡, significantly different from UTP stimulation ($p < 0.05$).

inhibitors on UTP-induced mucin secretion were investigated. NHBE cells were preincubated with various inhibitors for 15 min and then exposed to UTP (100 μM) plus the inhibitor for 2 h, and secreted mucin was measured by ELISA. The results indicated that mucin secretion provoked by UTP similarly required both PKC and PKG activities, as the secretory response was attenuated independently by the PKC inhibitor calphostin C (500 nM), the PKG inhibitor R_p-8-Br-PET-cGMP (10 μM), or the soluble guanylyl cyclase (GC-S) inhibitor LY83583 (50 μM) but not by the protein kinase A (PKA) inhibitor KT5720 (500 nM) (Fig. 1*C*). Apparently, mucin secretion in NHBE cells is regulated by a signaling mechanism involving both PKC and PKG.

MARCKS Is a Key Molecule Linking PKC/PKG Activation to Mucin Secretion in NHBE Cells

To address the signaling mechanism downstream of protein kinase activation, we turned our attention to a specific cellular substrate of PKC, MARCKS protein, that might play a role in linking kinase activation to granule release. We first confirmed the presence of MARCKS in NHBE cells by [^{3}H]myristic acid-labeled immunoprecipitation assay. As illustrated in Fig. 2*A*, MARCKS was expressed in NHBE cells, and the majority of this protein was membrane-associated under unstimulated conditions. Then a role for MARCKS as a key regulatory component of the mucin secretory pathway was demonstrated in three different ways.

Peptide Blocking Studies—NHBE cells were preincubated with either the MANS or the RNS peptide (1–100 μM) for 15 min, and then PMA (100 nM) + 8-Br-cGMP (1 μM) or UTP (100 μM) was added, and cells were incubated for an additional 15 min or 2 h, respectively. Mucin secretion was measured by ELISA. As shown in Fig. 2*B*, incubation of NHBE cells with the MANS peptide resulted in a concentration-dependent suppression of mucin secretion in response to PKC/PKG activation or UTP stimulation, whereas the control peptide (RNS) did not affect secretion at these same concentrations. Effects of the MANS peptide were not related to cytotoxicity or general repression of cellular metabolic activity, as neither the MANS nor the RNS peptide affected lactate dehydrogenase release or [^{3}H]deoxyglucose uptake by the cells (data not shown).

Antisense Oligonucleotide Studies—To demonstrate further MARCKS as a key signaling component of the mucin secretory pathway, the effect of an antisense oligonucleotide directed against MARCKS on mucin secretion was examined. As illustrated in Fig. 3, this antisense oligonucleotide down-regulated both mRNA and protein levels of MARCKS in NHBE cells and significantly attenuated mucin secretion induced by PMA + 8-Br-cGMP, whereas a control oligonucleotide had no effect.

Transient Transfection of HBE1 Cells—Transfection of HBE1 cells with the PSD-truncated MARCKS cDNA resulted in significant inhibition of mucin secretion in response to PMA + 8-Br-cGMP stimulation, whereas transfection with the wild-type MARCKS cDNA or the vector DNA had no effect (Fig. 4).

MARCKS Serves as a Convergent Signaling Molecule Mediating Cross-talk of PKC and PKG Pathways

To reveal molecular events by which MARCKS links kinase activation to mucin secretion, phosphorylation of MARCKS in response to PKC/PKG activation was investigated in depth. As illustrated in Fig. 5*A*, PMA (100 nM) induced a significant increase (3–4-fold) in MARCKS phosphorylation in NHBE cells, and this phosphorylation was attenuated by the PKC inhibitor calphostin C (500 nM). Once phosphorylated, MARCKS was

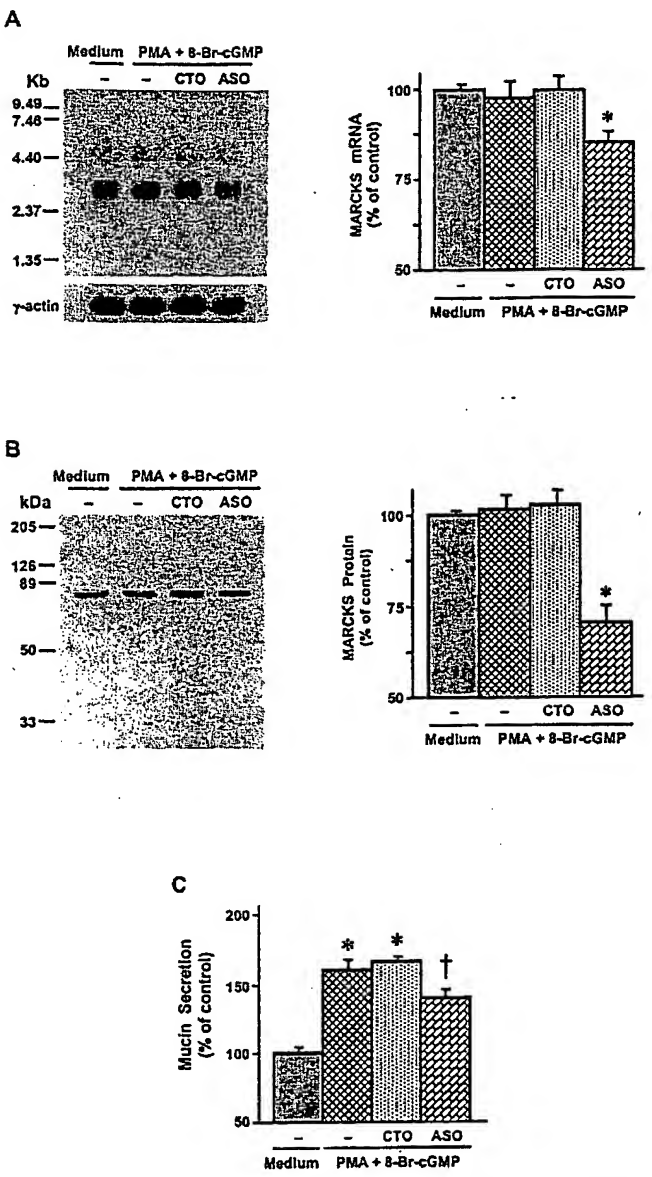

FIG. 3. **Antisense oligonucleotide directed against MARCKS down-regulates MARCKS expression and attenuates mucin hypersecretion.** NHBE cells were treated with the antisense or the control oligonucleotide for 3 days and then stimulated with PMA (100 nM) + 8-Br-cGMP (1 μM) for 15 min. Mucin secretion was analyzed by ELISA. Total RNA and protein were isolated from treated cells. MARCKS mRNA was assessed by Northern hybridization, and protein was assessed by Western blot. *A*, Northern blot showed a decrease of ~15% in MARCKS mRNA compared with controls. *B*, Western blot showed a decrease of ~30% in MARCKS protein. *C*, mucin hypersecretion was attenuated significantly by the antisense oligonucleotide, whereas the control oligonucleotide had no effect. Data are presented as mean ± S.E. (n = 6 at each point). *, significantly different from medium control ($p < 0.05$); †, significantly different from PMA + 8-Br-cGMP stimulation ($p < 0.05$). *CTO*, control oligonucleotide; *ASO*, antisense oligonucleotide.

translocated from the plasma membrane to the cytoplasm (Fig. 5*B*). Activation of PKG by 8-Br-cGMP (1 μM), another kinase activation event necessary for provoking mucin secretion, did not lead to MARCKS phosphorylation, but, in fact, the opposite effect was observed: MARCKS phosphorylation induced by PMA was reversed by 8-Br-cGMP (Fig. 6*A*). This effect of 8-Br-cGMP was not due to suppression of PKC activity, as the PMA-induced phosphorylation could be reversed by subsequent addition of 8-Br-cGMP to the cells (Fig. 6*B*). Therefore, PKG activation clearly resulted in dephosphorylation of MARCKS.

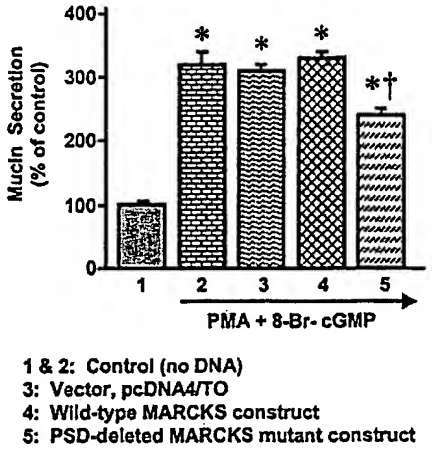

FIG. 4. **Transfection of PSD-deleted mutant MARCKS results in repression of mucin hypersecretion.** HBE1 cells were transiently transfected with wild-type MARCKS, PSD-deleted MARCKS, or vector DNA as described under "Experimental Procedures." After 48 h, cells were exposed to PMA (100 nM) + 8-Br-cGMP (1 μM) (*lanes 2–5*) or medium alone (*lane 1*) for 15 min. Mucin secretion was analyzed by ELISA. Data are presented as mean ± S.E. (n = 6 at each point). *, significantly different from medium control ($p < 0.05$); †, significantly different from PMA + 8-Br-cGMP stimulation ($p < 0.05$).

Further investigation showed that PKG-induced MARCKS dephosphorylation was blocked by 500 nM okadaic acid, a protein phosphatase (type 1 and/or 2A (PP1/2A)) inhibitor (Fig. 6*A*, *lane 6*). Thus, it appeared that the dephosphorylation was mediated by PP1 and/or PP2A. To define the subtype of protein phosphatase involved, a novel and more specific inhibitor of PP2A, fostriecin (IC_{50} = 3.2 nM) (20), was utilized in additional phosphorylation studies. As illustrated in Fig. 6*C*, fostriecin inhibited PKG-induced MARCKS dephosphorylation in a concentration-dependent manner (1–500 nM), suggesting that PKG induced the dephosphorylation via activation of PP2A. To confirm further activation of PP2A by PKG in NHBE cells, cytosolic PP1 and PP2A activities were determined after exposure of the cells to 8-Br-cGMP. PP2A activity was increased approximately 3-fold (from 0.1 to 0.3 nmol/min/mg proteins, $p < 0.01$) at concentrations of 8-Br-cGMP as low as 0.1 μM, whereas PP1 activity remained unchanged. These data clearly indicate that PP2A is activated by PKG and is responsible for the dephosphorylation of MARCKS. Accordingly, this PP2A activity appeared critical for mucin secretion to occur; when PKG-induced MARCKS dephosphorylation was blocked by okadaic acid or fostriecin, the secretory response to PKC/PKG activation or UTP stimulation was ameliorated (Fig. 7).

MARCKS Associates with Actin and Myosin in the Cytoplasm

Radiolabeled immunoprecipitation assay revealed that MARCKS associated with two other proteins (~200 and ~40 kDa) in the cytoplasm (Fig. 8). Matrix-assisted laser desorption ionization/time of flight mass spectrometry/internal sequence analysis indicated that these two MARCKS-associated proteins were myosin (heavy chain, non-muscle type A) and actin, respectively.

DISCUSSION

Transformed cell lines of airway epithelium tend to contain altered signaling pathways, and cell lines or nondifferentiated cells may not respond to exogenous stimuli in a manner similar to differentiated cells *in vivo*. The NHBE cells utilized in the present study were cultured at the air/liquid interface, resulting in fully differentiated primary cell cultures that maintained a well documented structure and function similar to the

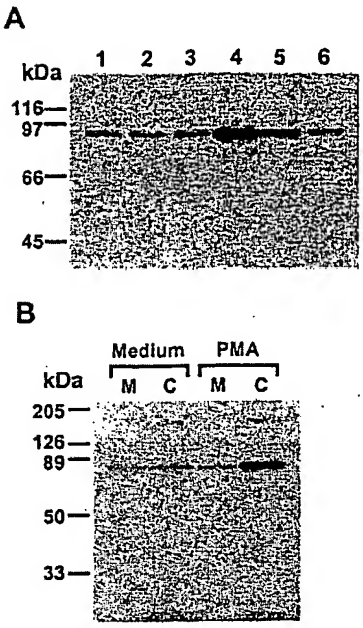

FIG. 5. **PKC-dependent phosphorylation releases MARCKS from the plasma membrane to the cytoplasm.** *A*, activation of PKC results in MARCKS phosphorylation in NHBE cells. Cells were labeled with [^{32}P]orthophosphate for 2 h and then exposed to the stimulatory and/or inhibitory reagents. MARCKS phosphorylation in response to the treatments was evaluated by immunoprecipitation as described. *Lane 1*, medium control; *lane 2*, the vehicle, 0.1% Me_2SO; *lane 3*, 100 nM 4α-PMA; *lane 4*, 100 nM PMA; *lane 5*, 100 nM PMA + 500 nM calphostin C; *lane 6*, 500 nM calphostin C. *B*, phosphorylated MARCKS is translocated from the plasma membrane to the cytoplasm. ^{32}P-Labeled cells were exposed to PMA (100 nM) or medium alone for 5 min, and then the membrane and the cytosol fractions were isolated. MARCKS phosphorylation in each fraction was evaluated by immunoprecipitation assay. *M*, membrane fraction; *C*, cytosolic fraction.

in vivo one (8, 21–23). This air/liquid methodology to culture airway epithelial cells was developed several years ago to provide an ideal *in vitro* model system to study mechanisms involved in various cellular processes in airway epithelium. The cell cultures contain secretory cells as well as ciliated and basal cells. Responses of these cells relate to the *in vivo* situation as they maintain the heterogeneous cell-cell contacts and polarized epithelial structure that no doubt influence their behavior *in situ*. Although MARCKS is likely present in non-secretory cells also, the clear and rapid causal associations between modifications of MARCKS and secretory outcomes suggest that mucin secretion is the direct effect of the MARCKS-related molecular events, as demonstrated in this study, occurring within the secretory cells.

Previous studies with transformed cell lines and airway epithelial cells isolated from various animal species have suggested involvement of PKC or PKG in mucin secretion (3–7). We report here for the first time that concurrent activation of both PKC and PKG maximized mucin secretion from differentiated NHBE cells, and activation of either kinase alone is not sufficient to elicit a robust secretory response. These results are in general agreement with most of the previous findings. We did document an enhanced secretory response to PMA alone (Fig. 1, *column 4*), although the magnitude of the response was much less than that observed by others (4) in a rat goblet-like cell line. In addition, although we have reported previously that a cGMP analogue could induce significant mucin secretion from cultured guinea pig tracheal epithelial cells (7), it should be noted that this response did not reach significant levels until 8 h of exposure. A secretory response with such a long lag period is unlikely to be a direct effect and probably involves *de novo* protein synthesis as opposed to release of

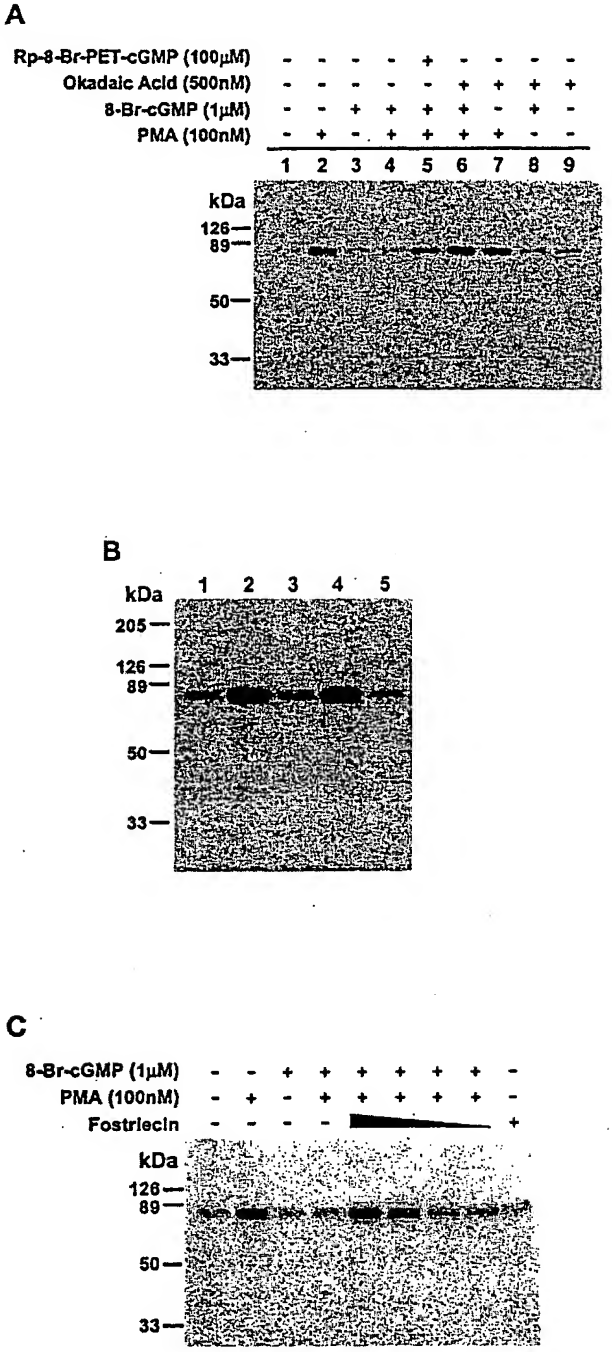

FIG. 6. **PKG induces dephosphorylation of MARCKS by activating PP2A.** NHBE cells were labeled with [^{32}P]orthophosphate and then exposed to the indicated reagents. MARCKS phosphorylation in response to the treatments was evaluated by immunoprecipitation assay. *A*, 8-Br-cGMP reversed MARCKS phosphorylation induced by PMA, and this effect of 8-Br-cGMP could be blocked by R_p-8-Br-PET-cGMP (PKG inhibitor) or okadaic acid (PP1/2A inhibitor). *B*, PMA-induced phosphorylation of MARCKS was reversed by subsequent exposure of cells to 8-Br-cGMP. *Lane 1*, medium alone for 8 min; *lane 2*, 100 nM PMA for 3 min; *lane 3*, 100 nM PMA for 3 min and then with 1 μM 8-Br-cGMP for 5 min; *lane 4*, 100 nM PMA for 8 min; *lane 5*, medium alone for 3 min and then 100 nM PMA + 1 μM 8-Br-cGMP for 5 min. *C*, 8-Br-cGMP-induced MARCKS dephosphorylation was attenuated by fostriecin in a concentration-dependent manner. ◣ from *left* to *right*: 500, 100, 10, and 1 nM fostriecin; the *last lane*: 500 nM fostriecin alone.

preformed and stored cytoplasmic granules. Nevertheless, the apparent synergistic effect involving cooperative activation of both PKC and PKG suggests a complex and stringent signaling mechanism mediating mucin secretion in differentiated human airway epithelium.

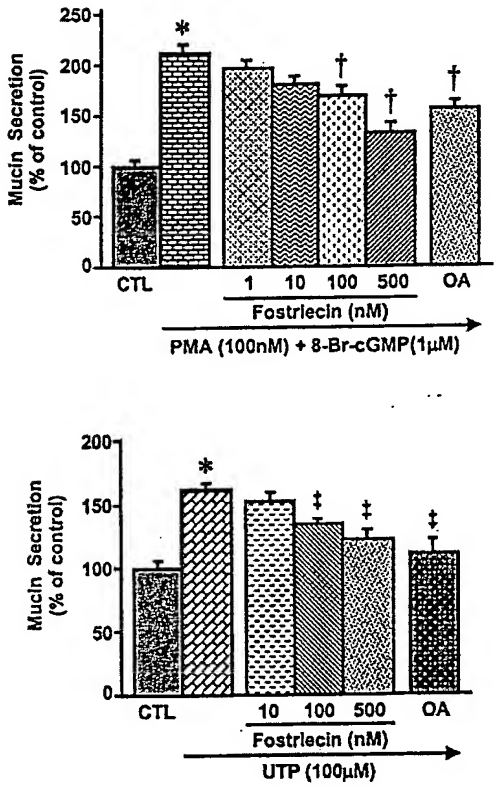

FIG. 7. **PP2A is an essential component of the mucin secretory pathway.** NHBE cells were preincubated with the indicated concentration of fostriecin, okadaic acid (500 nM), or medium alone for 15 min and then stimulated with PMA (100 nM) + 8-Br-cGMP (1 μM) for 15 min or with UTP (100 μM) for 2 h. Secreted mucin was measured by ELISA. Data are presented as mean ± S.E. ($n = 6$ at each point). *, significantly different from medium control ($p < 0.05$); †, significantly different from PMA + 8-Br-cGMP stimulation ($p < 0.05$); ‡, significantly different from UTP stimulation ($p < 0.05$).

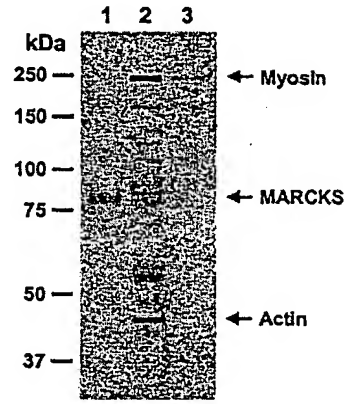

FIG. 8. **MARCKS associates with actin and myosin in the cytoplasm.** NHBE cells were labeled with [^{3}H]leucine and [^{3}H]proline overnight, and the membrane and the cytosol fractions were prepared as described under "Experimental Procedures." Isolated fractions were precleared with the nonimmune control antibody (6F6). The cytosol was then divided equally into two fractions and used for immunoprecipitation carried out in the presence of 10 μM cytochalasin D (Biomol, Plymouth Meeting, PA) with the anti-MARCKS antibody 2F12 (*lane 2*) and the nonimmune control antibody 6F6 (*lane 3*), respectively. MARCKS protein in the membrane fraction was also assessed by immunoprecipitation using the antibody 2F12 (*lane 1*). The precipitated protein complex was resolved by 8% SDS-polyacrylamide gel electrophoresis and visualized by enhanced autoradiography. MARCKS appeared to associate with two cytoplasmic proteins with molecular masses of ~200 and ~40 kDa, respectively. These two MARCKS-associated proteins were excised from the gel and analyzed by matrix-assisted laser desorption ionization/time of flight mass spectrometry/internal sequencing (the Protein/DNA Technology Center of Rockefeller University, New York). The obtained peptide mass and sequence data were used to search protein data bases via Internet programs ProFound and MS-Fit. Results indicate that they are myosin (heavy chain, nonmuscle type A) and actin, respectively.

To address involvement of PKG in the secretory process, 8-Br-cGMP was utilized in these studies. Although the primary physiological effect of 8-Br-cGMP is to activate PKG (24), it also has been reported to act as an agonist for cGMP-gated ion channels in some cells (25) and, at high concentrations, to cross-activate PKA (reviewed in Ref. 26). To preclude the possibility that cGMP-gated ion channels and/or PKA may play a role in mucin secretion by NHBE cells, R_p-8-Br-cGMP, a unique cGMP analogue that can activate cGMP-gated ion channels similar to 8-Br-cGMP but inhibit PKG activity (25), was used as an agonist to distinguish the effects of PKG and cGMP-gated ion channels on mucin release. As illustrated in Fig. 1*A* (*column 11*), R_p-8-Br-cGMP did not enhance mucin secretion when added to the cells with PMA. Likewise, the specific PKA inhibitor, KT5720 (500 nM), did not affect mucin secretion induced by either PMA + 8-Br-cGMP (data not shown) or UTP (Fig. 1*C*, *column 4*). These studies negate the possibility that cGMP-gated ion channels or PKA are associated with mucin secretion, indicating that activation of PKG in NHBE cells is the mechanism whereby 8-Br-cGMP contributes to enhanced secretion. Furthermore, because UTP-induced mucin hypersecretion can be attenuated by the soluble guanylyl cyclase (GC-S) inhibitor LY83583, it is likely that activation of PKG occurs via the signaling pathway of nitric oxide (NO) → GC-S → cGMP → PKG, as illustrated previously (7) in differentiated guinea pig tracheal epithelial cells *in vitro*.

Given the participation of both PKC and PKG in the mucin secretory process, we began to examine potential intracellular substrates of these enzymes that could play a role in signaling events downstream of the kinase activation. Numerous intracellular substrates can be phosphorylated by PKC or PKG, and phosphorylation by PKC of one such substrate, MARCKS protein, seemed to be of particular interest. The specific biological function of MARCKS is not clear; however, MARCKS phosphorylation has been observed to correlate with a number of cellular processes involving PKC signaling and cytoskeletal contraction, such as cell movement, mitogenesis, and neural transmitter release (reviewed in Ref. 27). Because the dynamic process of secretion requires both kinase activation and translocation of intracellular granules to the cell periphery, MARCKS appeared to be an ideal candidate for a mediator molecule connecting PKC/PKG activation and mucin granule exocytosis.

Direct involvement of MARCKS in mucin secretion by NHBE cells was demonstrated by three separate lines of evidence. First, mucin secretion in response to stimulation by PMA + 8-Br-cGMP or UTP was inhibited in a concentration-dependent manner by the MANS peptide, which had the amino acid sequence identical to the N-terminal region of MARCKS, whereas the corresponding control peptide (RNS), containing the same amino acid composition but arranged in random order, did not affect secretion. The N-terminal myristoylated domain of MARCKS is known to mediate the MARCKS-membrane association. As indicated in the hypothetical mechanism illustrated in Fig. 9, MARCKS may function as a molecular linker by interacting with granule membranes at its N-terminal domain and binding to actin filaments at its PSD site, thereby tethering granules to the contractile cytoskeleton for movement and exocytosis. It is also conceivable that attachment of MARCKS to the granules after it is released into the cytoplasm may be guided by specific targeting proteins or some other forms of protein-protein interactions in which the N-terminal domain of MARCKS is involved. In either case, the MANS peptide would

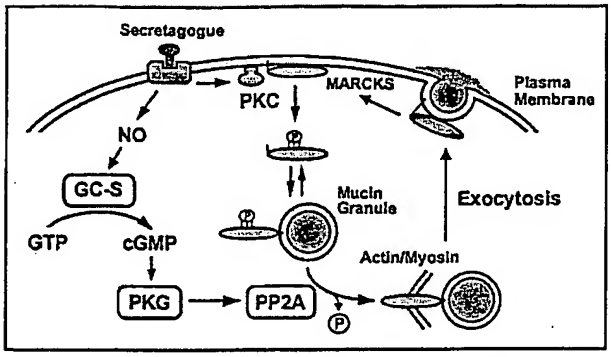

FIG. 9. **Hypothetical signaling mechanism controlling mucin secretion by human airway epithelial cells.** Mucin secretagogue interacts with airway epithelial (goblet) cells and activates two separate protein kinases, PKC and PKG. Activated PKC phosphorylates MARCKS, causing MARCKS translocation from the plasma membrane to the cytoplasm, whereas PKG, activated via the nitric oxide (NO) → GC-S → cGMP → PKG pathway, in turn activates a cytoplasmic PP2A, which dephosphorylates MARCKS. This dephosphorylation stabilizes MARCKS attachment to the granule membranes. In addition, MARCKS also interacts with actin and myosin, thereby linking granules to the cellular contractile machinery for subsequent movement and exocytotic release.

act to inhibit competitively targeting of MARCKS to the membranes of mucin granules, thereby blocking secretion.

The second line of evidence was provided by the inhibitory effect of a MARCKS-specific antisense oligonucleotide on mucin secretion. As shown in Fig. 3, the antisense oligonucleotide down-regulated MARCKS mRNA and protein levels in NHBE cells and substantially attenuated mucin secretion induced by PKC/PKG activation. The inhibition was not as dramatic as that seen with the MANS peptide, which might be due to the high levels of endogenous MARCKS protein in NHBE cells and the relatively long half-life of MARCKS mRNA ($t_{1/2}$ = 4–6 h) (28).

The final line of evidence was that transfection of HBE1 cells with a PSD-deleted mutant MARCKS resulted in significant repression of mucin secretion induced by PKC/PKG activation. Deletion of the PSD would abolish the ability of MARCKS to bind to actin. As indicated in Fig. 9, by competing with native MARCKS for binding to granule membrane, the PSD-truncated MARCKS could thereby inhibit granule release as it is unable to interact with the actin filaments. One piece of data that should be addressed here is the fact that transfection of these cells with the wild-type MARCKS cDNA did not further enhance mucin secretion. Western blot assay showed that the expression level of endogenous MARCKS in HBE1 cells was quite high, comparable with that in NHBE cells, and transfection of wild-type MARCKS cDNA did not lead to notable increases in overall MARCKS protein level in these cells (data not shown). This may explain why transfection with wild-type MARCKS did not further augment secretion and also why transfection with the PSD-deleted MARCKS only partially hindered mucin secretion.

Collectively, the above results demonstrated that MARCKS was involved integrally in the mucin secretory process. The next question to address was how MARCKS acts as a key regulatory molecule upon which PKC and PKG converge to regulate mucin secretion. A major part of the answer to this question could reside in the dynamics of MARCKS phosphorylation/dephosphorylation. As illustrated in Fig. 5, MARCKS was phosphorylated by PKC and consequently translocated from the membrane to the cytoplasm. Here, PKG appeared to induce dephosphorylation of MARCKS (Fig. 6*A*, *lane 4*, and Fig. 6*B*). This dephosphorylation was reversed by the PKG inhibitor R_p-8-Br-PET-cGMP (Fig. 6*A*, *lane 5*), indicating the dephosphorylation was specifically PKG-dependent.

How does PKG act to dephosphorylate MARCKS? An obvious mechanism is via activation of a protein phosphatase. As illustrated in Fig. 6*A* (*lane 6*), okadaic acid at 500 nM, a concentration that could inhibit both PP1 and PP2A, blocked PKG-induced dephosphorylation of MARCKS, suggesting that PKG caused dephosphorylation by activating PP1 and/or PP2A. Further studies with fostriecin and direct assay of phosphatase activities indicated that only PP2A was activated by PKG and was responsible for removal of the phosphate groups from MARCKS (Fig. 6*C*). Finally, to bring these results to a logical conclusion, either okadaic acid or fostriecin, at concentrations that inhibited PKG-induced dephosphorylation of MARCKS, attenuated mucin secretion induced by PMA + 8-Br-cGMP or UTP (Fig. 7). Thus, dephosphorylation of MARCKS by a PKG-activated PP2A appears to be an essential component of the signaling pathway leading to mucin granule exocytosis.

Results of these studies not only suggest a new paradigm for the signaling mechanism controlling exocytotic secretion of airway mucin granules but also provide the first direct evidence demonstrating a specific biological function of MARCKS in a physiological process; MARCKS serves as a key mediator molecule regulating mucin granule release in human airway epithelial cells. A hypothetical signaling pathway involving this secretory process is illustrated in Fig. 9. Specifically, elicitation of airway mucin secretion requires dual activation and synergistic actions of PKC and PKG. Activated PKC phosphorylates MARCKS, resulting in translocation of MARCKS from the inner face of the plasma membrane into the cytoplasm. Activation of PKG in turn activates PP2A, which dephosphorylates MARCKS in the cytoplasm. Because the membrane association ability of MARCKS is dependent on its phosphorylation state (9–11), this dephosphorylation would allow MARCKS to regain its membrane-binding capability and would enable MARCKS to attach to membranes of cytoplasmic mucin granules. By also interacting with actin and myosin in the cytoplasm (Fig. 8), MARCKS could then tether granules to the cellular contractile apparatus, mediating granule movement to the cell periphery and subsequent exocytotic release.

Certainly, the mechanism described above fits well into our current knowledge about mucin secretion and secretagogue activity. PKC and/or the NO → GC-S → PKG pathways have been thought to be involved in airway mucin secretion provoked by a wide range of stimuli. On a more etiological level, it also seems rational that a single intracellular molecule regulates the actual secretory event, that is movement and exocytosis of mucin granules. The need for activation of two separate protein kinases, which control phosphorylation and dephosphorylation of this single regulatory molecule, respectively, reflects another level of fine control of the secretory process. The wide distribution of MARCKS suggests the possibility that this or a similar mechanism may regulate secretion of membrane-bound granules in various cell types under normal or pathological conditions.

Acknowledgments—We thank Dr. Perry Blackshear from the NIEHS, Research Triangle Park, NC, for providing a number of MARCKS-related reagents and helpful advice.

REFERENCES

1. Adler, K. B., Fischer, B. M., Martin, L. D., and Voynow, J. A. (1998) *Res. Immunol.* **149**, 245–248
2. Cohn, L. A., and Adler, K. B. (1992) *Exp. Lung Res.* **18**, 299–322
3. Ko, K. H., Jo, M., McCracken, K., and Kim, K. C. (1997) *Am. J. Respir. Cell Mol. Biol.* **16**, 194–198
4. Abdullah, L. H., Conway, J. D., Cohn, J. A., and Davis, C. W. (1997) *Am. J. Physiol.* **273**, L201–L210
5. Abdullah, L. H., Davis, S. W., Burch, L., Yamauchi, M., Randell, S. H., Nettesheim, P., and Davis, C. W. (1996) *Biochem. J.* **316**, 943–951

6. Larivee, P., Levine, S. J., Martinez, A., Wu, T., Logun, C., and Shelhamer, J. H. (1994) *Am. J. Respir. Cell Mol. Biol.* **11**, 199–205
7. Fischer, B. M., Rochelle, L. G., Voynow, J. A., Akley, N. J., and Adler, K. B. (1999) *Am. J. Respir. Cell Mol. Biol.* **20**, 413–422
8. Krunkosky, T. M., Fischer, B. M., Martin, L. D., Jones, N., Akley, N. J., and Adler, K. B. (2000) *Am. J. Respir. Cell Mol. Biol.* **22**, 685–692
9. Seykora, J. T., Myat, M. M., Allen, L. A. H., Ravetch, J. V., and Aderem, A. (1996) *J. Biol. Chem.* **271**, 18797–18802
10. Swierczynski, S. L., and Blackshear, P. J. (1996) *J. Biol. Chem.* **271**, 23424–23430
11. McLaughlin, S., and Aderem, A. (1995) *Trends Biochem. Sci.* **20**, 272–276
12. Harlow, E., and Lane, D. (1988) *Antibodies: A Laboratory Manual*, pp. 570–573, Cold Spring Harbor Laboratory, Cold Spring Harbor, NY
13. Lin, H., Carlson, D. M., St. George, J. A., Plopper, C. G., and Wu, R. (1989) *Am. J. Respir. Cell Mol. Biol.* **1**, 41–48
14. Wright, D. T., Fischer, B. M., Li, C., Rochelle, L. G., Akley, N. J., and Adler, K. B. (1996) *Am. J. Physiol.* **271**, L854–L861
15. Spizz, G., and Blackshear, P. J. (1996) *J. Biol. Chem.* **271**, 553–562
16. Hartwig, J. H., Thelen, M., Rosen, A., Janmey, P. A., Nairn, A. C., and Aderem, A. (1992) *Nature* **356**, 618–622
17. Yankaskas, J. R., Haizlip, J. E., Conrad, M., Koval, D., Lazarowski, E., Paradiso, A. M., Rinehart, C. A., Jr., Sarkadi, B., Schlegel, R., and Boucher, R. C. (1993) *Am. J. Physiol.* **264**, C1219–C1230
18. Huang, X. C., Sumners, C., and Richards, E. M. (1996) *Adv. Exp. Med. Biol.* **396**, 209–215
19. Lethem, M. I., Dowell, M. L., Van Scott, M., Yankaskas, J. R., Egan, T., Boucher, R. C., and Davis, C. W. (1993) *Am. J. Respir. Cell Mol. Biol.* **9**, 315–322
20. Walsh, A. H., Cheng, A., and Honkanen, R. E. (1997) *FEBS Lett.* **416**, 230–234
21. Adler, K. B., Cheng, P. W., and Kim, K. C. (1990) *Am. J. Respir. Cell Mol. Biol.* **2**, 145–154
22. Kaartinen, L., Nettesheim, P., Adler, K. B., and Randell, S. H. (1993) *In Vitro Cell. Dev. Biol. Anim.* **29A**, 481–492
23. Gray, T. E., Guzman, K., Davis, C. W., Abdullah, L. H., and Nettesheim, P. (1996) *Am. J. Respir. Cell Mol. Biol.* **14**, 104–112
24. Sekhar, K. R., Hatchett, R. J., Shabb, J. B., Wolfe, L., Francis, S. H., Wells, J. N., Jastorff, B., Butt, E., Chakinala, M. M., and Corbin, J. D. (1992) *Mol. Pharmacol.* **42**, 103–108
25. Wei, J. Y., Cohen, E. D., Genieser, H. G., and Barnstable, C. J. (1998) *J. Mol. Neurosci.* **10**, 53–64
26. Schmidt, H. H., Lohmann, S. M., and Walter, U. (1993) *Biochim. Biophys. Acta* **1178**, 153–175
27. Aderem, A. (1992) *Trends Biochem. Sci.* **17**, 438–443
28. Harlan, D. M., Graff, J. M., Stumpo, D. J., Eddy, R. L., Jr., Shows, T. B., Boyle, J. M., and Blackshear, P. J. (1991) *J. Biol. Chem.* **266**, 14399–14405